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14. ABSTRACT: AIB1 (Amplified In Breast Cancer 1) is a nuclear receptor coactivator whose gene is amplified in 5-10% of breast cancers and both the mRNA and protein are overexpressed in ~30% of breast tumors. In vitro studies show that AIB1 plays a significant role in estrogen and IGF-1-induced cell proliferation. Germline knockout of the AIB1 gene leads to reduced somatic growth, abnormal reproductive function and reduced mammary gland development. Knockout of AIB1 expression also abrogates Ras-induced tumorigenesis. Furthermore, patients with tumors expressing high levels of the growth factor HER2/Neu in addition to AIB1 often develop anti-estrogen resistance to tamoxifen therapy. These findings imply that AIB1 plays a fundamental role in the development of hormone-independent breast cancer through growth factor mediated pathways. Nonetheless, the underlying mechanism of AIB1 regulation of growth factor mediated mammary neoplasia is unknown. In this investigation, I will utilize the MMTV-Neu mouse model (develop mammary gland tumors in 7-9 months) to elucidate the specific role of AIB1 in growth factor-induced mammary tumorigenesis.

15. SUBJECT TERMS
None provided.

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INTRODUCTION

The nuclear receptor coactivator 3 (NCOA3; AIB1/SRC-3) is the only steroid receptor coactivator (SRC) family member that is amplified and overexpressed in several types of human epithelial tumors, such as breast and prostate cancer (1-3). The *AIB1*/*SRC-3* gene is amplified on chromosome 20q in 5-10% of human breast cancers, and is overexpressed at both mRNA and protein levels in ~30% of breast cancer cell lines and breast tumors (1, 2, 4). The overexpression of AIB1/SRC-3 or AIB1-Δ3 (a potent isoform of AIB1) (5) in transgenic mice increased mammary epithelial cell proliferation, insulin-like growth factor (IGF-1) signaling and initiated the development of mammary hyperplasia and tumorigenesis (6, 7). SRC-3 knockout (SRC-$3^{-/-}$) mice display decreased mammary gland (MG) development during pregnancy, abnormal reproductive function, and MG growth retardation (8, 9). The loss of SRC-3 in MMTV/v-Ha-ras mice suppresses MG ductal hyperplasia, MG tumorigenesis and IGF-1 signaling (8-10). We have shown that loss of AIB1/SRC-3 in MCF-7 breast cancer cells decreases IGF-1 signaling, IGF-1 receptor expression levels, and IGF-1-induced anchorage-independent growth (11). Consistent with a central role for AIB1/SRC-3 in growth factor signaling, we recently reported that AIB1/SRC-3 knockdown by siRNA decreases epidermal growth factor (EGFR/HER1) phosphorylation, and EGFR-dependent downstream mitogenic signaling (12). Taken together, the observations in animal and *in vitro* models indicate that AIB1/SRC-3 plays a significant role in several growth factor-induced pathways that are relevant to breast cancer cell survival and proliferation.

One of the most important oncogenes in human breast cancer is the HER2/*neu* growth factor receptor tyrosine kinase, which belongs to the epidermal growth factor receptor (EGFR/HER) family. HER2/*neu* positive breast cancer responds to the monoclonal antibody trastuzumab, however, patients frequently develop resistance to the therapy. HER2/*neu* is amplified and overexpressed in 30% of human breast cancers and its expression is correlated with negative

prognosis and shortened disease-free survival (13). It has been reported that the overexpression of HER2/*neu* is correlated with high AIB1/SRC-3 mRNA levels in primary breast tumors (14). The overexpression of AIB1/SRC-3 was also found to be correlated with increased HER2/*neu* expression and resistance to tamoxifen in ER-positive breast cancer patients (15-17). These findings suggest that the biological roles of AIB1/SRC-3 and HER2/*neu* are linked in breast cancer and that AIB1/SRC-3 may increase the sensitivity of breast cancer cells to HER2/*neu*-driven tumorigenesis.

To assess the importance of the interplay between AIB1/SRC-3 and *Neu* in the development and progression of mammary cancer, I generated MMTV-*Neu*/SRC-$3^{+/-}$, and MMTV-*Neu*/SRC-$3^{-/-}$ mice. The MMTV-*Neu* mouse model overexpresses wildtype *Neu* in the MGs, resulting in an activating transmembrane mutation in the *Neu* transgene that promotes mammary tumorigenesis (18, 19). This model is ideal for studying the role of AIB1/SRC-3 in *Neu*-driven mammary tumorigenesis because it closely mimics the progression of human breast epithelial neoplasia, driven by the amplification and overexpression of the human homolog of HER2/*neu* (ErbB2) (13). Specifically, I want to determine whether the loss of AIB1/SRC-3 in MMTV-Neu mice alters the mammary gland morphology in MMTV-Neu transgenic mice. I propose that the loss of AIB1 will increase the latency and decrease the incidence of HER2/Neu-induced tumors.

BODY

Task 1 in my statement of work required the generation of female transgenic mice with the following genotypes: MMTV-*Neu*/SRC-3 wildtype (wt), MMTV-*Neu*/SRC-3 heterozygote (+/-), MMTV-*Neu*/SRC-3 knockout (-/-), as well as control litters MMTV-*Neu*, SRC-3^{wt}, SRC-$3^{+/-}$ and SRC-$3^{-/-}$ mice. In the previous report I discussed differences in primary and secondary branching of the mammary glands from the *Neu*/SRC-3^{wt}, *Neu*/SRC-3+/- and *Neu*/SRC-3-/- mice. The overexpression of the *Neu* oncogene in mice is also associated with extensive lateral side budding, an abnormal mammary phenotype partly driven by increased growth factor signaling (20). Thus the preneoplastic MG ductal morphology in female *Neu*/SRC-3^{wt}, *Neu*/SRC-$3^{+/-}$ and *Neu*/SRC-$3^{-/-}$ mice at 3-4 months of age was compared. Quantitative comparisons of the mammary gland whole mounts revealed a ~2 fold ($p<0.001$) and ~8 fold ($p<0.001$) decrease in lateral side budding in the *Neu*/SRC-$3^{+/-}$ and *Neu*/SRC-$3^{-/-}$ mice, respectively (Fig. 1*A* and 1*C*). Hematoxylin and eosin (H&E) stained sections also showed comparable differences in lateral side budding when SRC-3 levels were reduced in *Neu* mice (Fig. 1*A* lower panels).

Whole mount (WM) analysis at later stages of preneoplasia (6-7 months of age) revealed a considerable reduction (>60%, $p<0.001$) of lateral side budding in both the *Neu*/SRC-$3^{+/-}$ and *Neu*/SRC-$3^{-/-}$ mice relative to the *Neu*/SRC-3^{wt} mice (Fig. 1*B* and 1*C*). Consistent with previous reports using the SRC-$3^{-/-}$ mouse model, *Neu*/SRC-$3^{-/-}$ mice displayed decreased ductal branching (Fig. 1*A* and 1*B*, right panels), body weight and MG fat pad filling (data not shown) (8, 9). Interestingly, none of these effects was observed in the *Neu*/SRC-$3^{+/-}$ mice. Of note is that the *Neu*/SRC3^{wt} and *Neu*/SRC$3^{+/-}$ mice displayed no observable differences in ductal branching from 3-7 months of age.

Next, the relevance of SRC-3 in *Neu*-driven hyperplasia and tumor formation was investigated. Relative to the *Neu*/SRC-$3^{+/-}$ mice, *Neu*/SRC-3^{wt} mice, 9-12 months of age, displayed more extensive lateral side budding and the formation of hyperplastic alveolar nodules (HANs) in

the MGs adjacent to the mammary tumors (Fig. 2*A*). The *Neu*/SRC-3$^{-/-}$ mice displayed no discernible preneoplastic changes (Fig. 2*A*).

The appearance of palpable mammary tumors is shown in a Kaplan Meier plot of 25 *Neu*/SRC-3^{wt}, 23 *Neu*/SRC-3$^{+/-}$ and 14 *Neu*/SRC-3$^{-/-}$ mice (Fig. 2*B*). 77% of the *Neu*/SRC-3^{wt} mice developed mammary tumors from 7-15 months with a median age of 9 months. *Neu*/SRC-3$^{+/-}$ mice displayed a significant delay in tumor onset, with 70% of the mice developing mammary tumors from 15-24 months with a median age of 16 months. 100% of the *Neu*/SRC-3$^{-/-}$ mice observed up to 24 months of age were protected from mammary tumor formation (Fig. 2*B*). Despite the delayed tumorigeneis in the Neu/SRC3$^{+/-}$ mice there was no difference in the multiplicity of tumors per mouse (Fig 2*B* lower panel). It was also noted that *Neu*/SRC-3$^{+/-}$ tumors had large areas of necrotic tissue in all of the tumors examined (n=3 mice) (Fig. 2*C*). In contrast, all of the tumors from the *Neu*/SRC-3^{wt} mice displayed a noticeable increase in blood vessels when compared to the tumors from the Neu/SRC-3$^{+/-}$ mice (n=8 mice) (Fig. 2*C*). Relative to the Neu/SRC-3^{wt} mice, a ~20% ($p<0.05$) reduction was observed in the average number of blood vessels per field in the *Neu*/SRC-3$^{+/-}$ mice (Fig. 2*C*, bottom panel).

Currently, I am in the process of investigating changes in cell proliferation, cell cycle regulators (cyclin D1 and cyclin E) as well as how the reduction of SRC-3 mediates Neu receptor activation and Neu-induced downstream kinase signaling pathways (ie; MAPK, JNK and AKT). The mediation of signaling and proliferation will be investigated using immunohistochemical and western blot assays for the proteins of interest at both the pre-invasive (3-7 months) and neoplastic time points.

Figure 1. Decreased mammary gland lateral side budding in female MMTV-*Neu* mice with reduced levels of SRC-3 (*A* and *B*) Representative MG whole mount (WM, magnification: 4x) (top panels) and hematoxylin and eosin (H&E, magnification: 20x) (bottom panels) stained tissue sections from mice at (*A*) 3-4 and (*B*) 6-7 months of age. The data are representative of MGs taken from four mice from each genotype. Black arrows indicate lateral side budding in WM and H&E stained sections. (*C*) Quantification of lateral side budding along the MG ducts. The number of lateral side buds were counted from 10 fields per WM from each mouse (magnification: 10x). Data are expressed as the mean ± SD (n=4 mice from each genotype). *, $p< 0.05$ and **, $p<0.001$, one-way ANOVA.

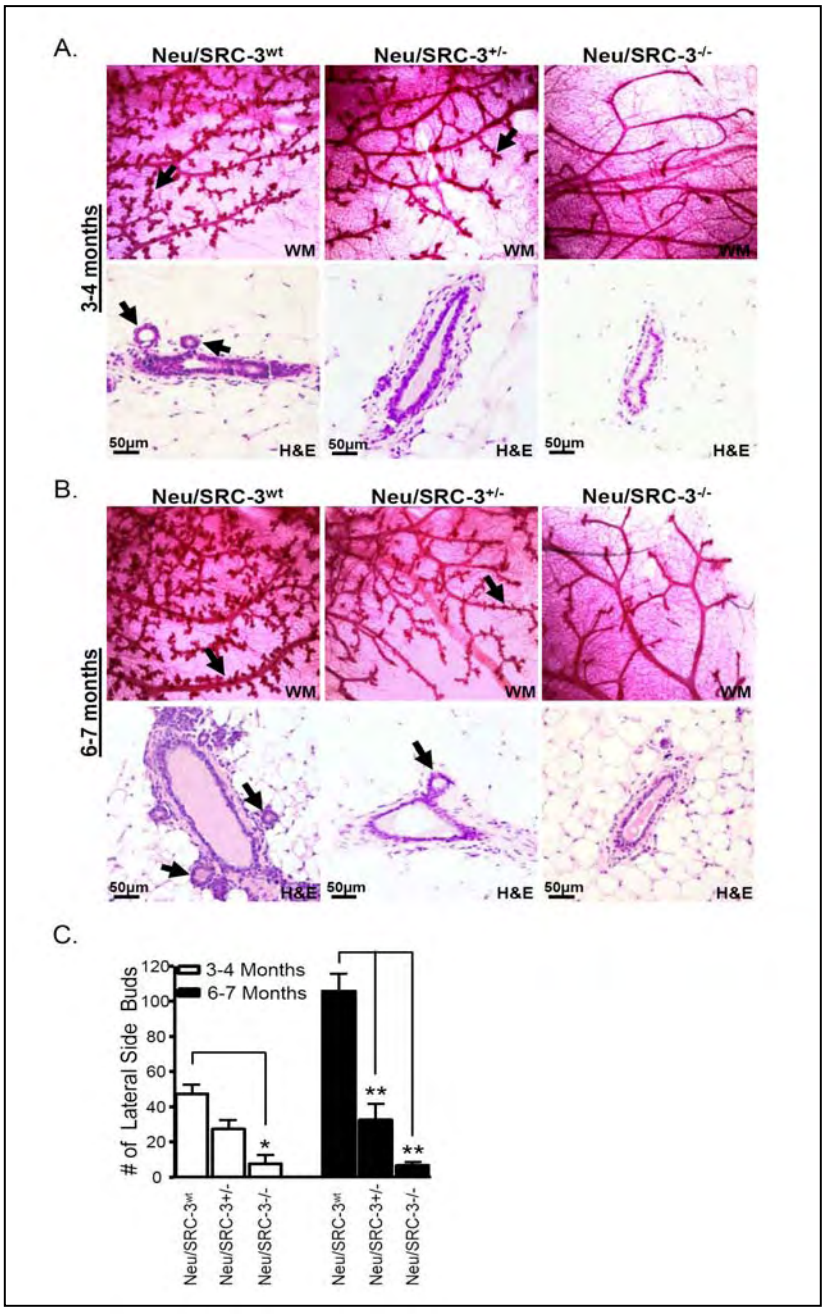
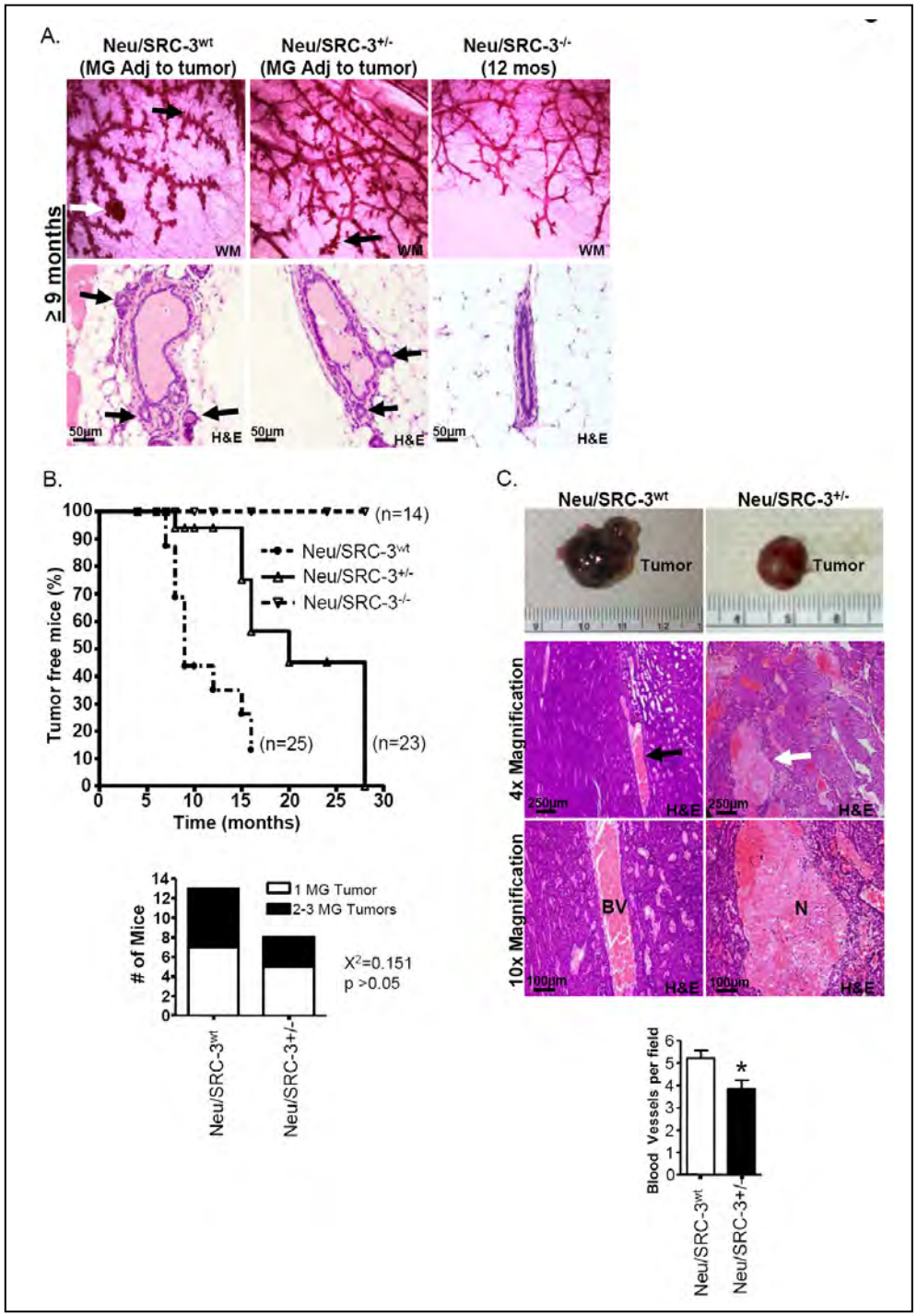


Figure 2. Reduced SRC-3 levels in MMTV-*Neu* mice increases the latency of *Neu*-driven mammary tumorigenesis (*A*) Representative MG whole mounts (WM, magnification: 10x) (top panels) and hematoxylin and eosin (H&E, magnification: 20x) (lower panels) stained tissue sections from MGs adjacent to tumors from *Neu*/SRC-3^{wt}, *Neu*/SRC-$3^{+/-}$, and *Neu*/SRC-$3^{-/-}$ mice. The data is representative of four mice examined from each genotype. Black arrows represent lateral side budding and the white arrow (top panel) indicates the presence of a typical hyperplastic alveolar nodule. (*B*, upper panel) Kaplan-Meier analysis of tumor-free incidence comparing *Neu*/SRC-3^{wt} with the *Neu*/SRC-$3^{+/-}$ and *Neu*/SRC-$3^{-/-}$ mice. (*B,* lower panel) Quantification of the number of tumors per mouse from the *Neu*/SRC-3^{wt} (n=8) and *Neu*/SRC-$3^{+/-}$ mice (n=13) was analyzed using the chi-squared test. (*C*) Photographs showing representative primary mammary tumors harvested from *Neu*/SRC-3^{wt} (9 months of age) and *Neu*/SRC-$3^{+/-}$ (19 months of age) mice (top panels). Representative paraffin-embedded H&E stained mammary tumors from *Neu*/SRC-3^{wt} and *Neu*/SRC-$3^{+/-}$ mice. Representative blood vessels (BV) (left panels) and areas of necrosis (N) (right panels). The bar chart represents the quantification of the average number of blood vessels per field in tumors from the *Neu*/SRC-3^{wt} (n=8) and *Neu*/SRC-$3^{+/-}$ (n=3) mice. 10 fields were counted per mouse (magnification: 40x). Data are expressed as mean ± SD. *, $p<0.05$, Student's t-test.


A.
Neu/SRC-3wt (MG Adj to tumor)
Neu/SRC-3+/- (MG Adj to tumor)
Neu/SRC-3-/- (12 mos)
≥ 9 months
WM
50μm
H&E
B.
Tumor free mice (%)
Time (months)
(n=14)
(n=25)
(n=23)
Neu/SRC-3wt
Neu/SRC-3+/-
Neu/SRC-3-/-
# of Mice
1 MG Tumor
2-3 MG Tumors
X2=0.151
p >0.05
Neu/SRC-3wt
Neu/SRC-3+/-
C.
Neu/SRC-3wt
Neu/SRC-3+/-
Tumor
4x Magnification
250μm
10x Magnification
BV
N
100μm
Blood Vessels per field
*
Neu/SRC-3wt
Neu/SRC-3+/-

KEY RESEARCH ACCOMPLISHMENTS

- Pathological analysis of wholemounts of the *Neu*/SRC-3^{wt}, *Neu*/SRC-3$^{+/-}$ and *Neu*/SRC-3$^{-/-}$ mice at 3-4, 6-7 months of age.
- Histological analysis of H&E stains of the *Neu*/SRC-3^{wt}, *Neu*/SRC-3$^{+/-}$ and *Neu*/SRC-3$^{-/-}$ mice at 3-4, 6-7 months of age.
- Pathological analysis of tumors from the *Neu*/SRC-3^{wt}, *Neu*/SRC-3$^{+/-}$.
- Histological analysis of H&E stains of the tumors from *Neu*/SRC-3^{wt}, *Neu*/SRC-3$^{+/-}$.
- Quantification of tumor-free incidence in *Neu*/SRC-3^{wt}, *Neu*/SRC-3$^{+/-}$ and *Neu*/SRC-3$^{-/-}$ mice

REPORTABLE OUTCOMES

This work has been presented and won the finalist award at Georgetown University's student research days poster competition.

CONCLUSIONS

In conclusion, this study demonstrates that SRC-3 plays a major role in *Neu*-induced preneoplastic phenotypic changes and tumorigenesis in the mammary gland of mice. Only a 50% reduction in SRC-3 levels in the mammary epithelium can delay HER2/*Neu*-driven tumorigenesis and reduce the total number of tumors that develop. In addition, the reduction in SRC-3 may reduce the angiogenesis in the tumors. Our results emphasize that targeting endogenous SRC-3 could provide greater efficacy to the current therapies used to treat tumors overexpressing HER2/*Neu*. I am currently collecting data to address which signaling cascades are mediating the phenotype observed in the Neu/SRC-3^{wt}, Neu/SRC-3$^{+/-}$ and Neu/SRC-3$^{-/-}$ mice.

ABBREVIATIONS

AIB1- Amplified in breast cancer 1
EGF- Epidermal growth factor
ER- Estrogen receptor
GR- Glucocorticoid receptor
GRIP1- Glucocorticoid receptor interacting protein 1
H&E- Hematoxillin and eosin
IGF- Insulin-like growth factor
IHC- Immunohistochemistry
MG- Mammary gland
MMTV- Mouse mammary tumor virus
PCNA- Proliferating cell nuclear antigen
PCR- Polymerase chain reaction
PPAR- Peroxisome proliferator-activated receptor
RAR- Retinoic acid receptor
rtTA- Reverse tetracycline transactivator
SRC- Steroid receptor coactivator

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